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USE OF A STRATIFIED OXIDATIVE STRESS MODEL TO STUDY THE BIOLOGICAL EFFECTS OF AMBIENT CONCENTRATED AND DIESEL EXHAUST PARTICULATE MATTER

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Although several epidemiological studies have shown a positive relationship between exposure to ambient air particulate matter (PM) and adverse health effects in humans, there is still a fundamental lack of understanding of the most toxic particle components and the biological mechanisms through which they act. Since our studies on the biological effects of diesel exhaust particles (DEP) have highlighted the role of reactive oxygen species (ROS), catalyzed by organic chemical compounds, we set out to establish whether this constitutes an oxidative stress model that can be used to study the biological effects of ambient coarse and fine PM. We demonstrate that organic DEP extracts induce a stratified oxidative stress response, leading to heme oxygenase 1 (HO-1) expression at normal GSH/GSSG ratios, proceed to Jun kinase activation and interleukin 8 (IL-8) production at intermediary oxidative stress levels, and culminate in cellular apoptosis in parallel with a sharp decline in GSH/GSSG ratios. We demonstrate that ambient concentrated air particulates, collected with a particle concentrator and a liquid impinger, mimic the effects of organic DEP extracts at lower oxidative stress levels. While fine PM consistently induced HO-1 expression in all most of the samples collected over a 9-mo survey period, coarse particulates were effective at inducing that effect during fall and winter. Moreover, HO-1 expression was positively correlated to the higher organic carbon (OC) and polyaromatic hydrocarbons (PAHs) content of fine versus coarse PM, as well as the rise in PAH content that occurs in coarse PM during the winter months. Although coarse and fine PM lead to a decrease in cellular glutathione (GSH)/GSSG ratios, oxidative stress did not increase to cytotoxic levels. Taken together, these data demonstrate that it is possible to use the stratified oxidative stress model developed for DEP to interpret the biological effects of coarse and fine PM. This work has important implications for the selection of relevant biological endpoints for in vivo studies.

Epidemiological studies have shown a positive relationship between exposures to ambient particulate matter (PM) and adverse health effects in susceptible human populations (Bates et al., 1995; Dockery et al., 1993; Pope et al., 1995; Sarnat et al., 2001). These adverse health effects include cardiopulmonary mortality and morbidity after a surge in PM_{10} (aerodynamic diameter < 10 μm) or $PM_{2.5}$ (aerodynamic diameter < 2.5 μm) levels. Although regulatory standards have been promulgated for PM_{10} and $PM_{2.5}$, there is still a fundamental lack of understanding of the most toxic particle constituents and the toxicological mechanisms through which they act (*Federal Register,* 1997; NRC, 1998). Research into these issues is vitally important to ascertain that nationwide control strategies regulate the actual toxicants responsible for adverse health effects (NRC, 1998). Suspended PM_{10} are complex aggregates of inorganic material (e.g., transition metals), salts (ammonium nitrate and sulfates), organic matter (carbonaceous compounds, including polyaromatic hydrocarbons), and proteinaceous compounds (Chow et al., 1994; Wall et al., 1988). While the coarse size fraction (2.5–10 μm) is dominated by natural sources (geogenic material: fugitive and resuspended dust; biological material: pollen and bacteria), fine particles (<2.5 μm) are dominated by anthropogenic emissions (Allen et al., 2000). The availability of ambient particle concentrators (Kim et al., 2001a, 2001b; Sioutas et al., 1995) and liquid impingers (Willeke et al., 1998; Zucker et al., 2000) now allow us to collect coarse and fine PM to study particle composition and toxicological effects.

Our own studies have concentrated on diesel exhaust particles (DEP) as a model anthropogenic pollutant that is responsible for allergic effects in the human respiratory tract (Nel et al., 1998; Diaz-Sanchez et al., 1997, 2000). Those studies have highlighted the role of reactive oxygen species (ROS), catalyzed by organic chemical compounds, in the proinflammatory effects of DEP (Hiura et al., 1999, 2000; Li et al., 2000; Nel et al., 2001). ROS exert a range of biological effects, including a change in the intracellular redox state, which leads to oxidative stress. Oxidative stress exerts proinflammatory effects in target cells in the respiratory tract, including macrophages and bronchial epithelial cells (MacNee & Donaldson, 1998; Bayram et al., 1998; Ohtoshi et al., 1998; Li et al., 1996; Becker et al., 1996; Goldsmith et al., 1997). These effects are mediated by phosphorylation-dependent cell signaling, including the involvement of stress-activated protein kinases and the NF-κB kinase cascade in the transcriptional activation of cytokine and chemokine genes that play a role in pulmonary inflammation and asthma (Nel et al., 1998; Takizawa et al., 1999; Sen & Packer, 1996; Lander, 1997; Folkard et al., 1997). In addition, we have demonstrated that crude methanol extracts made from DEP induce cellular apoptosis through an oxidative stress pathway that impacts the mitochondrial permeability transition (PT) pore (Hiura et al., 2000). In contrast to these adverse cellular effects, aromatic and polar organic chemical groups in DEP also induce cytoprotective effects (Li et al., 2000). One example is heme oxygenase 1 (HO-1) expression via a sensitive transcriptional activation pathway that regulates the promoter of that gene (Li et al., 2000; Prestera et al., 1995). Through its catalytic effects on heme, HO-1 generates the antioxidant bilirubin, as well as the gaseous substance CO (Maines, 1997). CO in the exhaled air is a sensitive in vivo marker for the proinflammatory effects of DEP in the lung (Nightingale et al., 2000). All considered, these studies indicate that DEP and organic DEP extracts that are enriched with polyaromatic hydrocarbons (PAHs) and oxy-PAHs induce a range of biological effects linked to their capacity to generate ROS. We propose that this range of effects constitutes a stratified cellular response to oxidative stress.

In this communication we set out to establish the principles of a stratified oxidative stress model, with a view to applying that model to the study of ambient coarse and fine particulates. Concentrated air particulates (CAPS) were collected directly into an aqueous medium, using a liquid impinger attached to the outflow of a particle concentrator (Sioutas et al., 1995; Kim et al., 2001a, 2001b; Willeke et al., 1998). These aqueous suspensions were reconstituted with powdered culture medium, and "real-life" particles incubated with macrophages, in parallel with DEP or organic DEP extracts. Our data demonstrate that fine particulates induce HO-1 expression more readily than coarse particles. The ambient PM effect on HO-1 was concordant with a relatively high organic carbon (OC) and PAH content in fine versus coarse particles. Both coarse and fine PM had little effect

on cellular toxicity, which occurs at the higher end of the oxidative stress scale using DEP extracts. Organic DEP extracts induced Jun kinase activation and interleukin 8 (IL-8) production at intermediary levels of oxidative stress.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, penicillin–streptomycin, amphotericin B, L-glutamine, and fetal calf serum (FCS) were purchased from GIBCO-BRL (Baltimore, MD). Diesel exhaust particles (DEP) were a generous gift of Dr. Masaru Sagai (National Institute of Environment Studies, Tsukuba, Ibaraki, Japan). These particles were collected onto glass-fiber filters from the exhaust from a 7JB1 low-duty Isuzu diesel engine under a load of 10 torque. Particles were scraped as a powder that is stored under N_2 gas in a glass container. *N*-Acetylcysteine (NAC), propidium iodide (PI), reduced and oxidized glutathione (GSH and GSSG), reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH), monoclonal anti-actin antibody (Ab), and glutathione reductase were obtained from Sigma Chemicals (St. Louis, MO). Monoclonal anti-HO-1 Ab were purchased from Stressgen (Victoria, Canada). Polyclonal anti-Mn superoxide dismustase (SOD) antiserum was obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies for phospho-JNK and JNK immunoblotting were obtained from two sources: anti-phospho-JNK_1 (p45) and anti-JNK_1 antisera were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-JNK (p45 and p54) and anti-JNK antisera recognizing JNK_1 and JNK_2 isoforms were from Cell Signaling Technology (Beverly, MA). Rabbit anti-mouse Ab, swine anti-rabbit Ab, and avidin–biotin complex were purchased from Dako (Carpinteria, CA). ECL reagents were obtained from Pierce (Rockford, IL). Annexin-FITC kit was purchased from Trevigen (Gaithersburg, MD).

Ambient Particle Collection for In Vitro Tests

Ambient coarse (2.5–10 μm) and fine (0–2.5 μm) particles were collected outdoors in the facilities of Rancho Los Amigos National Rehabilitation Center in Downey, CA, during the period 15 March to 7 December 2000. Situated near the Alameda corridor, Downey has some of the highest inhalable respirable particle concentrations (PM_{10}) in the United States, very often exceeding the National Ambient Air Quality Standard of 150 $\mu g/m^3$. Particle collections were conducted by the Versatile Aerosol Concentration Enrichment Systems (VACES), using three parallel sampling lines (concentrators) to simultaneously collect coarse and fine particles at a flow rate of 110 L/min. The VACES is described in greater detail by Kim et al. (2001a, 2001b). Coarse particles were concentrated using a single nozzle

virtual impactor, while fine particles were concentrated by drawing air samples through two parallel lines, using 2.5-μm cut-point preimpactors to remove larger sized particles. These particles are drawn through a saturation-condensation system that grows particles to 2- to 3-μm droplets, which are subsequently concentrated by virtual impaction.

Highly concentrated liquid suspensions of these particle modes were obtained by connecting the concentrated output flow from each of the VACES concentrators to a liquid impinger (BioSampler, SKC West, Inc., Fullerton, CA) (Figure 1). Particles are injected into the BioSampler in a swirling flow pattern so that they can be collected by a combination of inertial and centrifugal forces. Under normal operating conditions (at its nominal flow rate of 12.5 L/min), the BioSampler has collection efficiency close to 100% for particles larger than about 1.5 μm. For particles smaller than 1.0 μm, the collection efficiency decreases sharply to less than 50% (Willeke et al., 1998). Operating in conjunction with the VACES, however, the BioSampler can collect any of the PM size ranges with 100% efficiency and at a sampling flow rate that is at least 10-fold higher than its nominal operating flow rate. Thus, the supersaturational growth of even ultrafine PM to supermicrometer particles enables effective trapping of these particles by the impinger, and allows us to "concentrate" large numbers of ambient PM into a very small solution (i.e., on the order of 5–10 ml) of water. Ten concurrent sets of coarse and fine PM were collected during sampling runs requiring 6–7 h to collect 2 mg PM. Before collection of each sample, the BioSampler was autoclaved.

Chemical Analysis of Concentrated Particles

For chemical analysis, two reference samples were collected: The first was a collection of coarse and fine particles on Teflon filters (47 mm, PTFE,

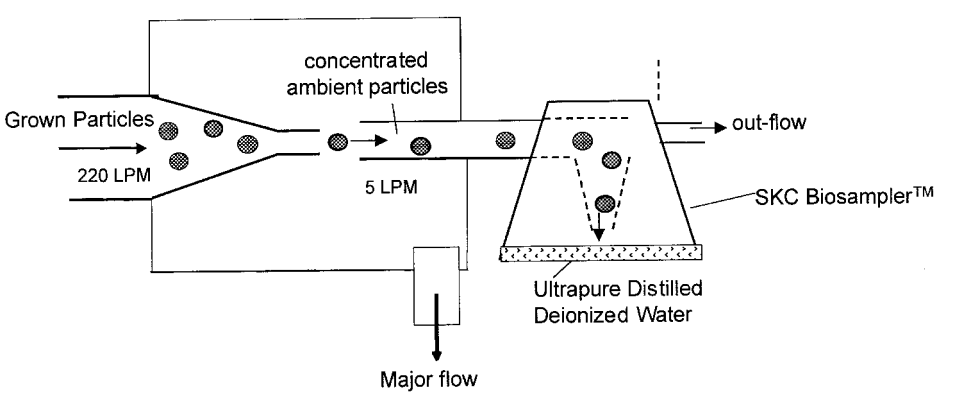

FIGURE 1. Schematic to illustrate use of the particle concentrator to collect ambient coarse and fine PM for in vitro tests. Particles are first concentrated by an approximate factor of 40, from airflow of 220 L/min to airflow of 5 L/min. Following concentration enrichment, particles are captured in the BioSampler by combined impaction and centrifugation.

2 μm pore, Gelman Science, Ann Arbor, MI), using the microorifice uniform deposit impactor (MOUDI, MSP Corp.; Marple et al., 1991). Mass concentrations were determined by weighing the MOUDI before and after each field test in a Mettler 5 microbalance (MT 5, Mettler-Toledo, Inc., Highstown, NJ). Filters were weighed after a 24-h equilibration time period. Laboratory and field blanks were used for quality assurance. The Teflon filters were then analyzed by means of ion chromatography to determine the concentrations of particulate sulfate and nitrate. The second collection was done on two 47-mm quartz filters (Pallflex Corp., Putnam, CT), using the USC dichotomous sampler as described by Misra et al. (2001). These filters were used for measurement of trace element and metal concentrations, as well as for determining PAH, elemental carbon (EC), and organic carbon (OC) concentrations. A slice of approximately 0.2 cm^2 from each filter was placed in a platinum boat containing MnO_2. The sample was acidified with an aliquot of HCl and heated to 115°C to form CO_2 as an index of particle-associated carbon. The boat was then inserted into a dual zone furnace, where MnO_2 oxidized OC at 550°C and EC at 850°C. A flame ionization detector converted the CO_2 combustion product to CH_4 for detection. The remaining filter was divided in two equal parts; one half was analyzed by means of x-ray fluorescence for measurement of trace element and metal concentrations. The other half was analyzed by a high-performance liquid chromatography (HPLC) fluorescence method for detection of a group of 16 signature PAHs as previously described (Li et al., 2000). These included: naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benz[a]anthracene (BAA), chrysene (CRY), benzo[b]fluoranthene (BBF), benzo[k]fluoranthene (BKF), benzo[a]pyrene (BAP), indeno[1,2,3-cd]pyrene (IND), dibenz[a,h]anthracene (DBA); benzo[ghi]perylene (DGB).

DEP Fractionation and GC/MS Analysis

The DEP extract was from 1 g particles using 60 ml methylene chloride in a Virtis homogenizer (Li et al., 2000). The combined extracts were concentrated by roto-evaporation, and asphaltenes were precipitated by exchanging into hexane. The supernatant was concentrated, dried over anhydrous sodium sulfate, and subjected to silica-gel column chromatography (column size 1 × 30 cm) following the method of Venkatesan et al. (Li et al., 2000; Venkatesan et al., 1980). Aliphatic, aromatic, and polar fractions were collected by elution with 20 ml hexane, 40 ml 3:2 hexane:methylene chloride, and 30 ml 1:1 methylene chloride:methanol, respectively. The fractions were weighed in a microbalance by evaporating off a known volume of an aliquot of the sample made up in methylene chloride or methanol.

Cell Culture and Stimulation

RAW 264.7 and THP-1 cells were cultured in DMEM or RPMI 1640, respectively, supplemented by 1% penicillin/streptomycin and 10% fetal

calf serum (FCS) (complete medium). DEP extracts were prepared as previously described (Hiura et al., 1999, 2000; Li et al., 2000). Briefly, 100 mg DEP was suspended in 25 ml methanol and sonicated for 30 s. The DEP/methanol suspension was centrifuged at 2000 rpm for 10 min at 4°C. The methanol supernatant was transferred to a preweighed polypropylene tube and dried under nitrogen gas (Hiura et al., 1999, 2000; Li et al., 2000). Dried DEP extracts were resuspended in dimethyl sulfoxide (DMSO) at 100 μg/μl and stored at –20°C in the dark. The NAC stock solution (1 *M*) was made in HEPES immediately before use, and diluted to a final concentration of 20 m*M* in culture medium. For exposure to whole DEP or DEP extracts, cells were plated at 2×10^6/well in 6-well plates, and the particles or extracts added to the indicated final concentrations in 3 ml of complete medium. Controls were treated with DMSO at a final concentration of 0.1%. All cell cultures were maintained in a 37°C humidified incubator supplemented with 5% CO_2.

The medium used to study the effects of ambient particles was prepared in the following way: Aqueous particle suspensions were replenished with DMEM powder according to the manufacturer's instructions and supplemented with 10% FCS and a 1:200 dilution of penicillin/streptomycin/amphotericin B. For the studies using ambient particles, RAW 264.7 cells were seeded at 0.67×10^6/well in 12-well plates containing 1 ml of complete medium. Control cells were cultured in basal DMEM containing the same amount of antibiotics/antimycotics and FCS.

Western Blotting

The cells were harvested by scraping and lysed as previously described (Li et al., 2000). One hundred micrograms of total lysate protein was electrophoresed on sodium dodecyl sulfate (SDS) polyacrylamide gels before transfer to PVDF membranes. To determine HO-1 expression, blots were sequentially overlayed with anti-HO-1 mAb at 0.3 μg/ml and a 1:1000 dilution of rabbit anti-mouse Ab conjugated to horseradish peroxidase (HRP) (Li et al., 2000). MnSOD immunoblotting was conducted with polyclonal anti-MnSOD Ab (0.3 μg/ml). Biotinylated swine anti-rabbit Ab (1:1000) was used as secondary Ab, followed by HRP-conjugated avidin–biotin complex (1:10000). Western blotting of phospho-JNK and the various JNK isoforms was performed using rabbit antisera (1:1000), followed by HRP-conjugated goat anti-rabbit Ab (1:1000). For actin immunoblotting, stripped membranes were overlayed with monoclonal anti-actin Ab (1:200), followed by HRP-conjugated rabbit anti-mouse Ab (1:1000). All blots were developed with the ECL reagent according to the manufacturer's instructions.

Determination of GSH/GSSG Ratio

Total glutathione (GSH plus 50% of GSSG) and glutathione disulfide (GSSG) were measured using recycling assays involving the reaction of 5,5′-dithio-bis(2-nitrobenzoic acid) and glutathione reductase (Tietze, 1969; Griffith, 1980; Anderson, 1985). Briefly, cells were lysed and deproteinized

in 3% 5-sulfosalicylic acid (SSA). Whole-cell lysates were then cleared by centrifugation at 4°C at 14,000 rpm in an Eppendorf centrifuge. The supernatant was used for the measurement of total and oxidized glutathione. The amount of total glutathione, expressed as micromoles per 10^6 cells, was calculated from a reduced glutathione (GSH) standard curve prepared in SSA. For GSSG assay, 100 µl of supernatant was incubated with 2 µl 2-vinylpyridine (2-VP) and 6 µl triethanolamine for 60 min on ice. GSSG standards were treated in the same way as samples. The amount of GSSG in the samples was calculated from the GSSG standard curve. The amount of reduced GSH was calculated by subtracting the amount of GSSG from that of total glutathione.

Apoptosis Assays

Apoptosis was determined by flow cytometry using the annexin-FITC kit according to the manufacturer's instruction (Hiura et al., 1999, 2000). Briefly, 0.5×10^6 cells were resuspended in 500 µl binding buffer containing 1 µl annexin V and 10 µl PI supplied by the manufacturer. The cells were incubated with annexin V and PI for 30 min at room temperature in the dark prior to the analysis. Flow cytometry was performed using a FACScan (Becton Dickenson, Mountain View, CA) equipped with a single 488-nm argon laser (Hiura et al., 1999, 2000). The fluorescence of annexin V and PI were analyzed with emission settings of 525 nm (FL-1) and 575 nm (FL-2), respectively.

Statistical Analysis

Biological data were analyzed using SAS statistical software (SAS Institute, Inc., Cary, NC). Scheffé's method of multiple comparisons with *F*-test was used for ANOVA. Particle data were assessed by the Excel 5.0 statistical package, using Student's *t*-test.

RESULTS

Exposures to Organic DEP Extracts Induce a Dose-Dependent Decline in Cellular GSH/GSSG Ratios

The intracellular ratio of reduced (GSH) to oxidized (GSSG) glutathione is a tightly regulated homeostatic process that serves to maintain intracellular redox balance (Rahman et al., 1995). A drop in the intracellular GSH/GSSG ratio, such as occurs when ROS are being produced by redox cycling of PAHs and oxy-PAHs, signals the cell that it has become oxidatively stressed (Hiura et al., 1999; Rahman et al., 1995; Kumagai et al., 1997). This may initiate protective responses or, if the level of oxidative stress is more acute, could induce injurious effects (Rahman et al., 1995; Nel et al., 2001). Exposure of RAW 264.7 and THP-1 macrophage cell lines to incremental amounts of a methanol DEP extract induced a dose-dependent de-

cline in the intracellular GSH/GSSG ratios in both cell types (Figure 2). Thus, while both cell types maintained a normal glutathione ratio in the presence of 1–10 μg/ml of DEP extract, there was a gradual but reproducible decline in GSH/GSSG ratios in the presence of 10–50 μg/ml extract, followed by a steep decline at doses >50 μg/ml (Figure 2). This provided us with a dose-dependent profile against which to compare biological responses associated with oxidative stress.

HO-1 and MnSOD Expression Is Induced by Organic DEP Extracts at the Lower End of the Oxidative Stress Scale

We have previously demonstrated that crude organic DEP extracts, as well as aromatic and polar DEP fractions that are enriched for PAHs and oxy-PAHs, respectively, induce HO-1 expression in tissue culture cells (Li et al., 2000). HO-1 expression is a sensitive oxidative stress marker, which is mediated by the antioxidant response element (ARE) in the HO-1 promoter (Prestera et al., 1995; Keyse & Tyrrell, 1989). Exposure of THP-1 and RAW

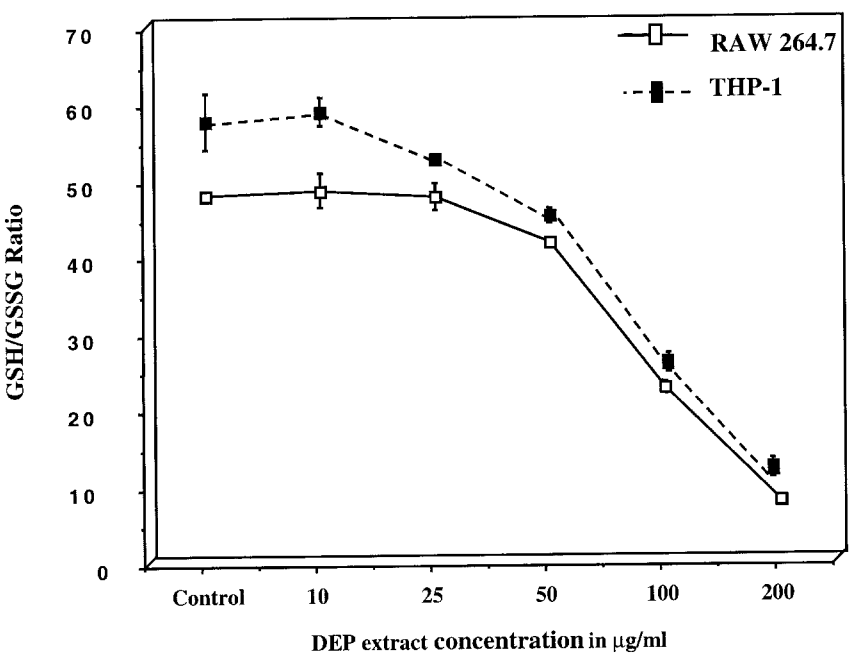

FIGURE 2. Treatment of macrophage cell lines with a crude organic DEP extract induces a dose-dependent decline in cellular GSH/GSSG ratios. Duplicate aliquots of RAW 264.7 and THP-1 were treated with the indicated amounts of a crude methanol DEP extract for 8 h, and the cellular lysates were used for measuring reduced (GSH) and oxidized (GSSG) glutathione levels as described in Materials and Methods. Error bars represent SEM. This experiment was reproduced twice. The decrease in the GSH/GSSG ratios are statistically significant at an extract dose of >50 μg/ml (p = .001). Control GSH values were 199.12 ± 6.3 and 402.2 ± 27.4 μmol/10^6 cells for RAW 264.7 and THP-1, respectively.

264.7 cells, to as little as 1 μg/ml of the DEP extract elicited HO-1 expression (Figure 3A). The magnitude of the response increased as the extract dose was increased, reaching a plateau at 100 μg/ml (Figure 3A). Through its catalytic effect on heme, HO-1 exerts antioxidant and cytoprotective effects in the lung (Otterbein et al., 1999; Willis et al., 1996). Moreover, another antioxidant enzyme, MnSOD, was induced by the same extract, starting at a dose as low as 2.5 μg/ml (Figure 3B). This effect was specific since the expression of a household protein, β-actin, did not change during DEP exposure (Figure 3B, lower panel). Taken together, these results show that MnSOD and HO-1 expression are sensitive oxidative stress markers, which respond to ROS generation even before there is glutathione generation.

Evidence that Organic DEP Extracts Induce JNK Activation and IL-8 Production at Intermediary Levels of Oxidative Stress

The same organic extract used in Figure 2 induced a dose-dependent increase in 45-kD JNK (JNK_1) phosphorylation, starting at doses as low as 2.5 μg/ml and reaching a plateau at 10 μg/ml (Figure 4A, upper panel). There

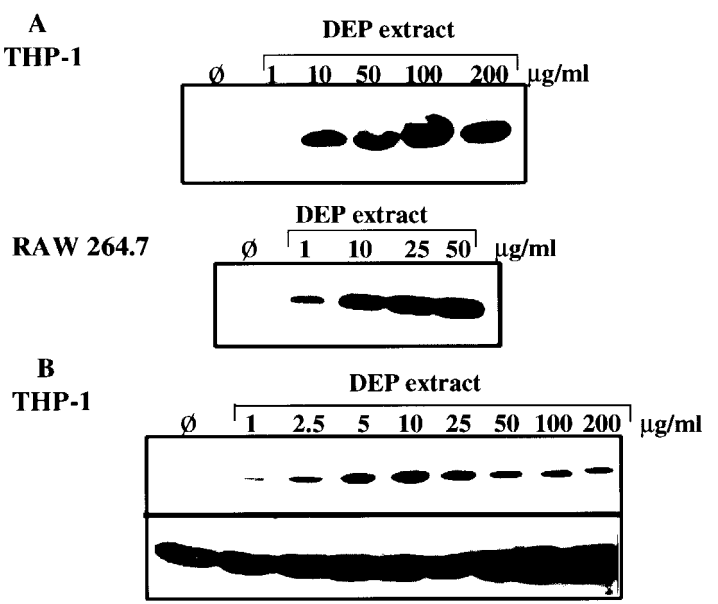

FIGURE 3. Immunoblotting studies showing dose-dependent induction of HO-1 and MnSOD expression in macrophage cell lines treated with an organic DEP extract. RAW 264.7 and THP-1 cells were treated with the indicated amounts of the organic DEP extract used in Figure 2 for 7 h before cellular extraction and resolving the proteins by 10% SDS polyacrylamide gel electrophoresis (PAGE). After transfer to PVDF membranes, blots were overlayed with (A) HO-1 and (B) MnSOD antibodies as described in Materials and Methods. The blot shown in (B) was stripped and overlaid a second time with anti-β-actin Ab to demonstrate there is no change in this cellular household protein during extract exposure. These results were reproduced twice.

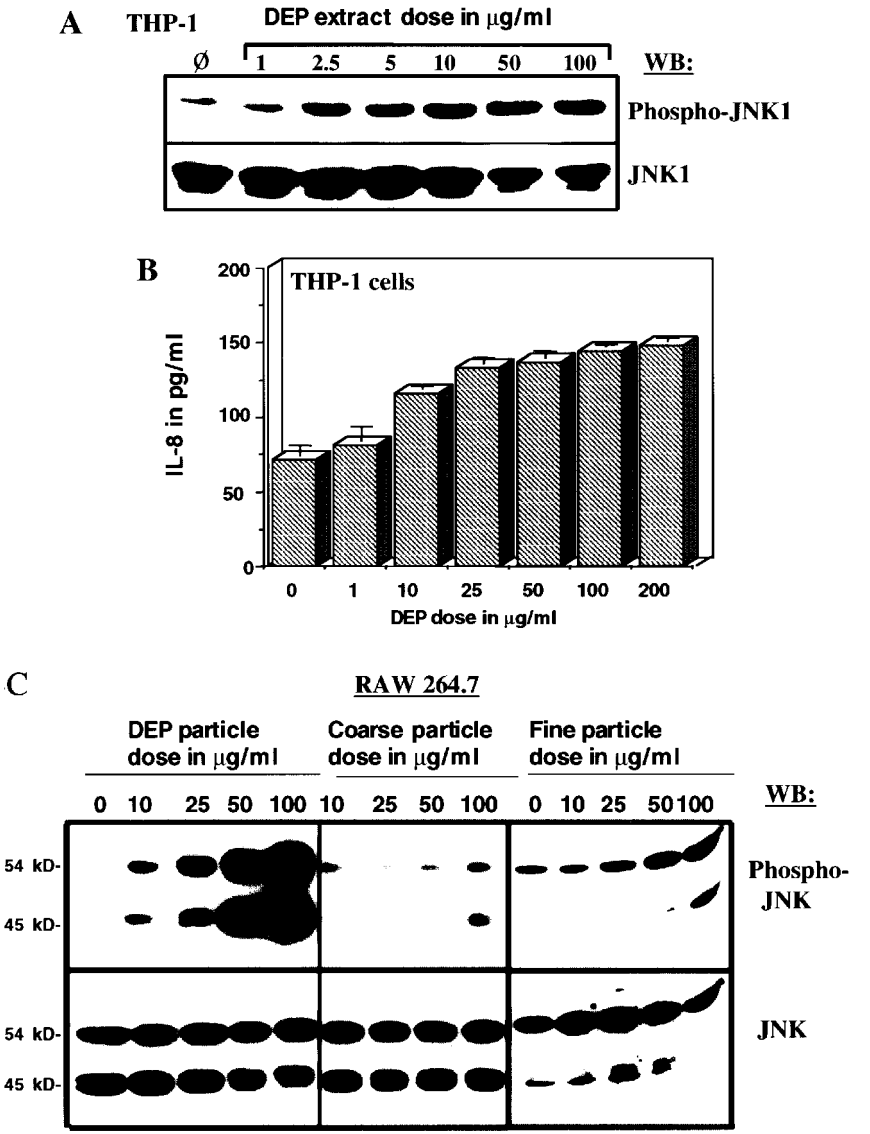

FIGURE 4. A crude DEP extract induce dose-dependent JNK_1 activation and IL-8 production in THP-1 cells. (A) THP-1 cells were exposed to the indicated amount of the DEP extract for 30 min, before cellular lysates were subjected to phospho-JNK_1 and JNK_1 (Santa Cruz Biotechnology) immunoblotting as described in Materials and Methods. (B) Duplicate aliquots of THP-1 cells were treated with the indicated amounts of the DEP extracts for 16 h before collection of the cellular supernatants, which were frozen and sent to Cytokine Laboratories (Baltimore, MD) for measurement of IL-8 levels by an ELISA assay. This experiment was reproduced once. (C) RAW 264.7 cells were exposed to DEP, coarse or fine ambient particles, at indicated concentrations, for 90 min. Western blotting was performed using anti-phospho-JNK_1/JNK_2 and anti-JNK_1/JNK_2 antibodies (Cell Signaling Technology) as described in the Materials and Methods.

was no change in the level of JNK_1 expression (lower panel), which is compatible with the fact that increased JNK_1 phosphorylation leads to kinase activation without changing the abundance of this enzyme (Kyriakis et al., 1994). The same results, using whole DEP and antisera that recognize both JNK_1 and JNK_2 isoforms, were obtained in RAW 264.7 cells (Figure 4C). Both isoforms were activated in a dose-dependent fashion by the intact DEP (Figure 4C). Since the JNK cascade initiates biological effects via the activation of AP-1 response elements, we studied the expression of a chemokine, IL-8, that has been linked to JNK activation and that has been shown to be involved in the proinflammatory effects of DEP in vivo and in vitro (Ng et al., 1998; Ohtoshi et al., 1998; Mori et al., 1998; Lakshminarayanan et al., 1998; Salvi et al., 2000). Exposure of THP-1 cells to extract doses >10 μg/ml induced IL-8 secretion (Figure 4B). This coincided with the plateau in the JNK response (Figure 4A), as well as a phase of gradual decline in cellular GSH/GSSG ratios (Figure 2). Murine cells do not produce IL-8, and IL-8 levels were not measured in the supernatant of RAW 264.7 cells.

Evidence that Organic DEP Extracts Induce Cytotoxicity at the Upper End of the Oxidative Stress Scale

We have previously shown that DEP and crude DEP extracts induce cytotoxicity in macrophages through perturbation of the mitochondrial PT pore (Hiura et al., 1999, 2000). That leads to cytochrome *c* release and initiation of cellular apoptosis, which can be assayed by two-color annexin V/PI staining and flow cytometry (Hiura et al., 1999, 2000). We have also demonstrated that this programmed death response evolves through a number of distinguishable phases, beginning with membrane asymmetry (annexin V^+ staining) at an early apoptotic phase, followed by annexin V^+ and PI^+ staining (late apoptosis), and ultimately cellular apoptosis and necrosis (annexin V^-/PI^+ staining) (Hiura et al., 1999, 2000). Utilizing the same extract employed in Figures 2–4, we demonstrated a dramatic decrease in cell viability (white bars) at extract doses >25 μg/ml (Figure 5). This is accompanied by a dose-dependent increase in the number of dead cells (Figure 5). These cytotoxic effects were coincident with the dose leading to a steep decline in cellular GSH/GSSG ratios (Figures 2 and 5). Taken together with the data in Figures 2–4, these data provide evidence for a stratified cellular response, in which cytoprotective effects dominate at low oxidative stress levels, then yield to proinflammatory and ultimately cytotoxic effects at higher oxidative stress levels. This provides a useful reference for studying the biological effects of coarse and fine PM.

CAPs Induce Oxidative Stress as Determined by the Decline in the Intracellular GSH/GSSG Ratios

Fine (<2.5 μm) and coarse (2.5–10 μm) particles were collected in the Alameda corridor by two parallel sampling lines (concentrators) that connect the output to a liquid impinger (BioSampler) (Figure 1). After reconstitution

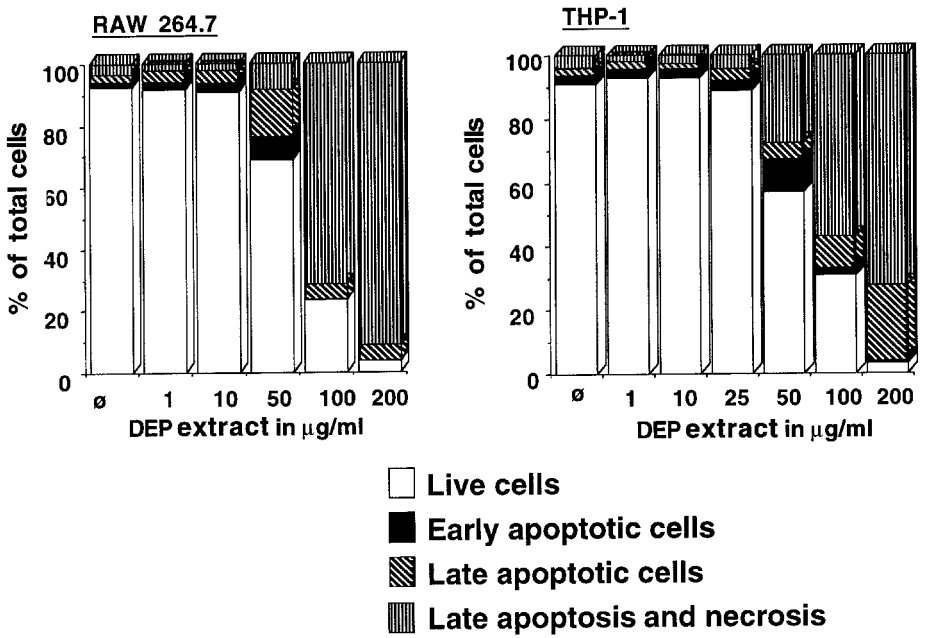

FIGURE 5. Dose-dependent induction of cellular apoptosis in macrophages cell lines by crude DEP extracts. RAW 264.7 and THP-1 cells were exposed to the extracted amounts of the crude DEP extracts for 18 h before harvesting and staining of the cell pellets with annexin V and PI as described in Materials and Methods. Cells were analyzed in a FACScan (Becton Dickenson) to determine percent of cells in each population that are annexin V and PI negative (live cells), annexin V positive and PI negative (early apoptotic cells), annexin V and PI positive (late apoptotic cells), or annexin V negative but PI positive (late apoptosis and necrosis). This experiment was reproduced twice with identical results.

of the aqueous samples in complete culture medium, RAW 264.7 cells were exposed to whole DEP coarse and CAPS in tissue culture plates. Our data show that intact DEP, similar to extracts from these particles, induced a dose-dependent decline in cellular glutathione ratios (Figure 6, experiments 1 and 2). Compared to a negligible effect of the DEP extract dose of 10 μg/ml (Figure 2), whole particles induced a statistically significant decrease in the GSH/GSSG ratios at a comparable dose ($p < .001$). This could be due to more effective cellular uptake of the particles compared to the extract. While fine particles also showed a significant ($p < .05$) and dose-dependent response, the rate of decrease in the GSH/GSSG ratio was not as precipitous as with DEP (Figure 6, experiment 1). Coarse particles, on the other hand, did not induce a statistically significant effect on glutathione ratios, only yielding a modest decline at a concentration of 50–100 μg/ml (Figure 6, experiment 2). These data suggest differences in the potency of these particulates to generate oxidative stress. This notion was further confirmed by looking at HO-1 expression (discussed below) and immunoblotting to study JNK activation (Figure 4C). Thus, while whole DEP induced a robust and

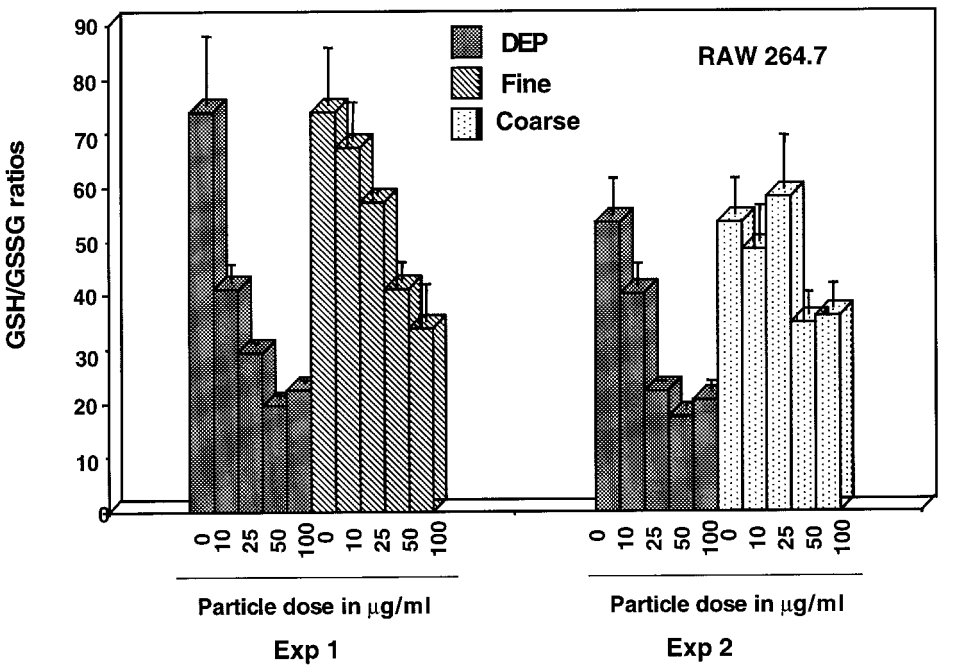

FIGURE 6. Assessment of glutathione ratios in RAW 264.7 cells treated with whole DEP, fine and coarse particles. Duplicate aliquots of cells were treated with the indicated amounts of whole DEP, coarse and fine CAPS for 8 h in 2 separate experiments, before measurement of intracellular GSH and GSSG levels as described in Figure 2.

dose-dependent activation of the 45- and 54-kD JNK isoforms, fine particulates exerted an intermediary effect, whereas coarse particles elicited a poor response (Figure 4C). It was not possible to study JNK activation by CAPS in THP-1 cells due to lack of enough particle material. RAW 264.7 cells were therefore used for the in vitro CAPS analyses, as well as for comparison with lung tissue from intact mice (ongoing studies).

Differences in the Chemical Composition of Ambient Coarse and Fine Particulates

Figure 7 shows the average chemical composition of coarse and fine particles based on 10 field measurements over a 9-mo study period. Almost 60% of the coarse PM mass was comprised of crustal elements (Figure 7), which is reflected by the finding that 22% of coarse PM mass was associated with Si in the detailed chemical profile (Figure 8A). Additional crustal metals, such as Mg, Al, Ca, and K account for ~35% of coarse PM mass (Figure 8A). In contrast, trace metals that are generated by incomplete combustion processes, such as Ti, Mn, Zn, Cu, and Ba, only comprised 1.5% of coarse PM mass (Figure 8A). Approximately 20.2 (SD of ± 7.8)% of coarse PM mass was associated with the organic carbon (OC) fraction (Figure 7A).

This includes 31.3 (± 8.9) ng/mg PAH per coarse PM mass (Figure 8B). Acenaphthene, phenanthrene, pyrene, and benzo[ghi]perylene were the most abundant PAH, with mass fractions varying from 3 to 7 ng/mg of PM mass (Figure 8B). No measurable amounts of naphthalene were found in either coarse or fine particles. The remaining coarse PM mass was associated with nitrate (mostly sodium nitrate, as opposed to ammonium nitrate found in fine PM), and sulfate, with mass fractions of 14.7 (± 3.8)% and 7.3 (± 2.3)%, respectively (Figure 7). Coarse PM contains very little elemental carbon.

OC is by far the most abundant component of fine PM, accounting for 45.3 (± 10.7)% of the total PM mass, whereas EC accounted for about 6.2 (± 1.3)% of the mass (Figure 7). In accordance with the relatively high OC content, detailed chemical analysis revealed that PAH are more abundant in fine than coarse particles (Figure 8B). The total average PAH content was 138 (± 38) ng/mg of fine particle mass, which is approximately 4 times higher than in coarse particles. Phenanthrene, pyrene, fluoranthene, benzo-[a]pyrene and benzo[ghi]perylene are the most abundant PAH, with mass fractions varying from 8 to 30 ng/mg PM (Figure 8B). Fine also differed from coarse particles in a lower metal content (16.6%; Figure 7B). Figure 8A plots the mass fractions of each metal. Sulfur is the most abundant element in fine PM, mainly associated with sulfate salts. Crustal elements, such as Al, Si, Ca, and K, are also present, but the mass fractions are four to five times smaller than that of coarse PM. Fe is also present in fine PM, but only contributing 2.6% of the total, which is about one-fourth of the Fe contribution to coarse PM. The mass fractions of combustion elements, such as Ti,

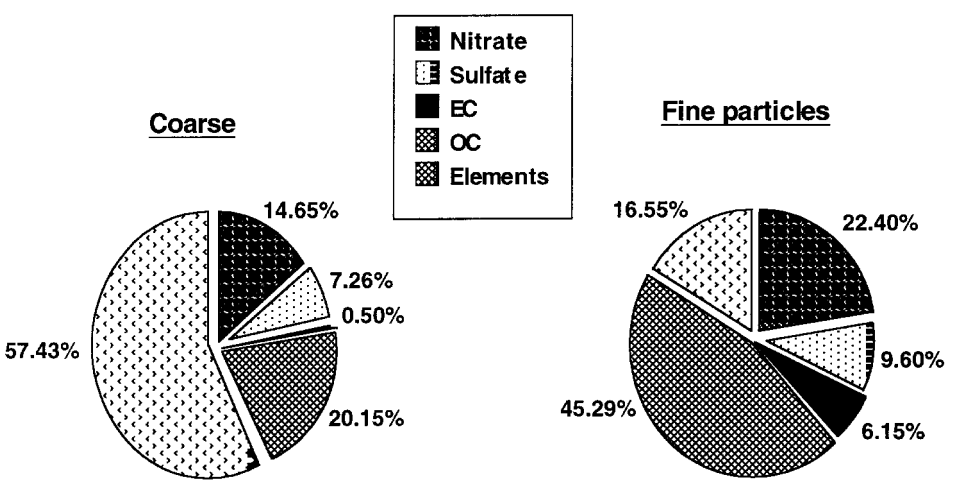

FIGURE 7. Average fractional composition of coarse and fine particulates collected over a 9-mo time period. Ten batches of coarse and fine PM were collected concurrently between March and December 2000 in Downey, CA. These particles were subjected to chemical analysis as described in Materials and Methods, and the percent for each component was calculated. Standard deviations are given in the text.

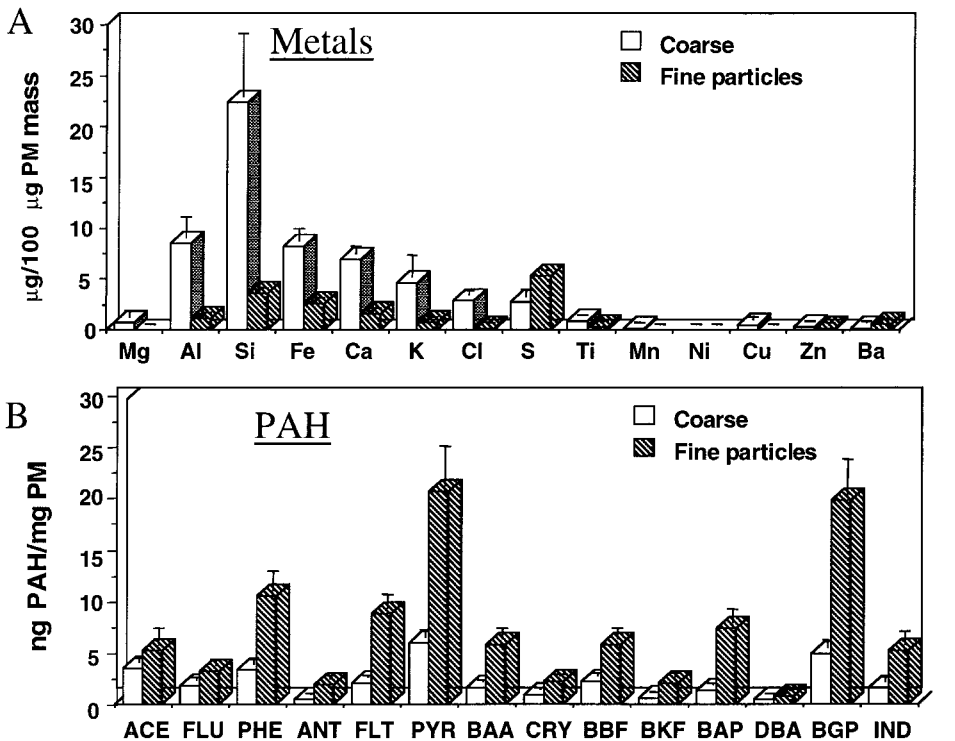

FIGURE 8. Average PAH and metal content for the 10 coarse and fine PM batches described in Figure 7. CAPS were collected on filters, which were used to determine the detailed elemental and PAH composition as described in Materials and Methods. The abbreviations are listed in the same section. Error bars represent standard deviations.

Ni, Zn, and Ba, in fine PM mass are comparable to those found for coarse PM. Inorganic ammounium salts, such as ammonium nitrate and sulfate, account for approximately one-third of the total fine PM mass, with the mass fractions of nitrates and sulfate being 22.4 (± 5.7)% and 9.6 (± 2.8)%, respectively (Figure 7).

Differences in the Oxidative Stress Effects of Coarse and Fine Particles as Determined by HO-1 Expression

We used the stratified oxidative stress model outlined in Figures 2–5 as a grid for the comparing biological effects of ambient coarse and fine particles. CAPS were incubated with RAW 264.7 cells in tissue culture plates, and the recovered cell pellets were used for conducting HO-1 immunoblots. Intact DEP or organic chemical groups fractionated from crude DEP extracts were used as positive controls. Comparison of six matched pairs of coarse and fine PM revealed differences in coarse versus fine chemical composition (Table 1) and potency for HO-1 expression. In the representative samples shown in Figures 9–12, fine particles induced HO-1 expression on every test

occasion (March, June, September, December), while coarse particles only exerted effects during the latter two occasions (Figures 11 and 12). It is noteworthy that HO-1 expression was accompanied by a fractional OC ratio ≥29% on all occasions where fine and coarse particles successfully induced this response, except for September 2000 (Table 1 and Figures 9–12). Since the OC fraction includes the PAHs, the association of this chemical group to HO-1 expression is in accordance with our hypothesis that redox cycling PAHs are involved in the oxidative stress effects leading to the activation of the HO-1 promoter. That notion is supported by the ability of aromatic and polar DEP fractions, which are enriched for PAHs and quinones, respectively, to induce HO-1 expression (Figure 9). Moreover, a surge in the OC and PAH content in coarse particles during December 2000 may explain HO-1 expression on that occasion (Figure 12). This prompted us to collect further coarse PM samples during the winter time period (January/February 2001), which revealed that three out of four batches induced HO-1 expression (Figure 13). Moreover, the PAH contents of these coarse particles were even higher than for the December 2000 collection (Figure 13). This represents a statistically significant increase in the PAH content in winter (January/February) compared to summer (June/July) months ($p < .001$). More direct proof for the role of oxidative stress in HO-1 expression was provided by ability of *N*-acetylcysteine to interfere with the effect of fine (Figure 10) and coarse particles (not shown) in vitro. Fine and coarse particles also induced MnSOD expression (data not shown).

Coarse and Fine Particles Have Limited Cytotoxic Effects

While clearly effective for inducing HO-1 expression, both sources of CAPS were considerably less potent then DEP in inducing apoptosis, as determined by two-color annexin V/PI flow cytometry (Figures 9–12). Coarse particles were slightly more toxic than fine particles (Figures 9–12), with the exception of the December 2000 PM collections (Figure 12). These data suggest that ambient particles collected in aqueous media do not achieve the high levels of oxidative stress that is required to induce programmed cell death.

TABLE 1. Fractional chemical analysis of coarse and fine particulates (as percent of total PM mass)

	March 2000		June 2000		September 2000		December 2000	
	Coarse	Fine	Coarse	Fine	Coarse	Fine	Coarse	Fine
Nitrate	14	29	14	19	27	22	7	22
Sulfate	7	8	7	26	6	4	9	6
EC	1	6	<1	4	1	8	<1	9
OC	16	38	9	29	15	55	36	45
Elements	62	19	70	22	51	11	48	21

Note. Chemical analysis was performed on coarse and fine PM collected on Teflon and quartz filters as described in Materials and Methods.

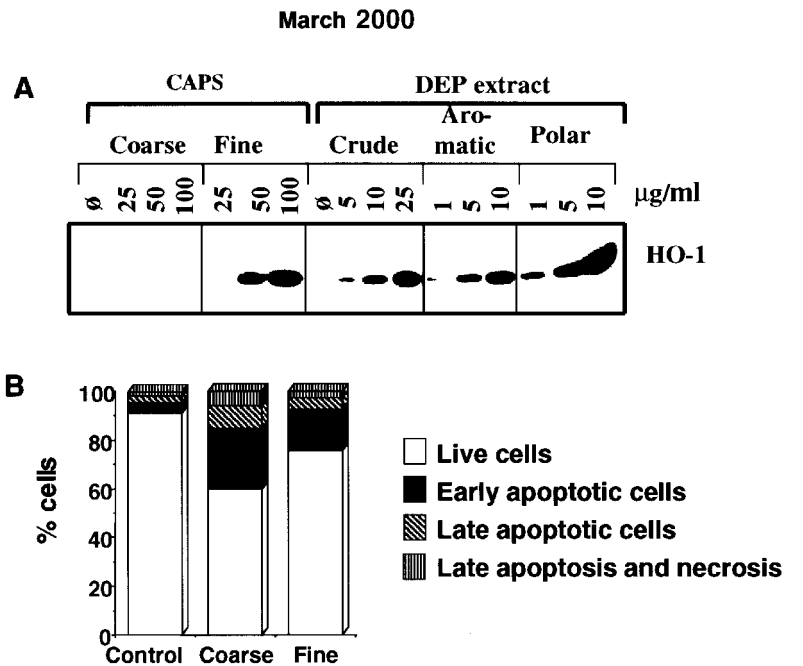

FIGURE 9. Comparison of coarse and fine PM effects on HO-1 expression and a flow cytometry assay for apoptosis in March 2000. (A) Concentrated coarse and fine PM, collected in a BioSampler, were incubated with RAW 264.7 for 16 h at the indicated concentrations. For comparison, we also used the indicated amounts of a crude DEP extract, as well as aromatic and polar fractions prepared from a methylene chloride DEP extract by silica gel chromatography, as previously described (Li et al., 2000; Venkatesan et al., 1980). We have demonstrated that the aromatic fraction is enriched with PAHs and nitro-PAH but does not contain quinones, while the polar fraction is enriched for quinones but does not contain any of the signature PAHs (Li et al., 2000). (B) Two-color annexin V/PI flow cytometry was performed on the cell pellet of RAW 264.7 cells exposed to 300 µg/ml coarse and 300 µg/ml fine PM for 18 h. The performance and interpretation of this assay are the same as for Figure 5.

DISCUSSION

In this article we show that organic DEP extracts induce a stratified oxidative stress response that begins with HO-1 expression at normal GSH/GSSG ratios, proceeds to JNK activation and IL-8 secretion at intermediary oxidative stress levels, and culminates in cellular toxicity at high oxidative stress levels. We demonstrate that ambient CAPS, collected with a liquid impinger, mimic the effects of organic DEP extracts at lower oxidative stress levels. While fine particulates consistently induced HO-1 expression during the entire survey period, coarse particulates were more effective at inducing the same effect during the fall and winter months. Moreover, this biological effect was positively correlated to the higher OC and PAH content of fine versus coarse particles, as well as the rise in PAH content that occurs in coarse particles during the winter months. Although coarse and fine particles induce oxidative stress, their oxidative stress levels were not

high enough to commit the cells to an apoptotic fate. Taken together, these data demonstrate that it is possible to use the stratified oxidative stress model to study the differential toxicity of coarse and fine PM in vitro.

These data are of considerable importance in terms of the research priorities defined by the Committee on Research Priorities for Airborne Particulate Matter (PM), which was formed in 1998 by the National Academy of Sciences, on the request of the U.S. Environmental Protection Agency (NRC, 1998). Among the 10 highest research priorities listed, work in this article addresses one of the most important issues, namely, "Investigations into toxicologic mechanisms by which ambient airborne PM produces mortality and morbidity" (NRC, 1998). In this communication we show that there is a possible mechanistic link between the high OC content of fine and, occasionally, coarse PM and HO-1 expression in vitro. The exquisite sensitivity of this response to oxidative stress in vitro (Keyse & Tyrrell, 1989), is also reflected by in vivo data showing that CO levels in expired air are a sensitive marker for the effects of DEP inhalation under experimental conditions (Nightingale et al., 2000). CO is a catabolic product formed by the enzymatic activity of a family of heme oxygenases (Maines, 1997). The inducible isoform, HO-1, is expressed in the lung and is the likely source of

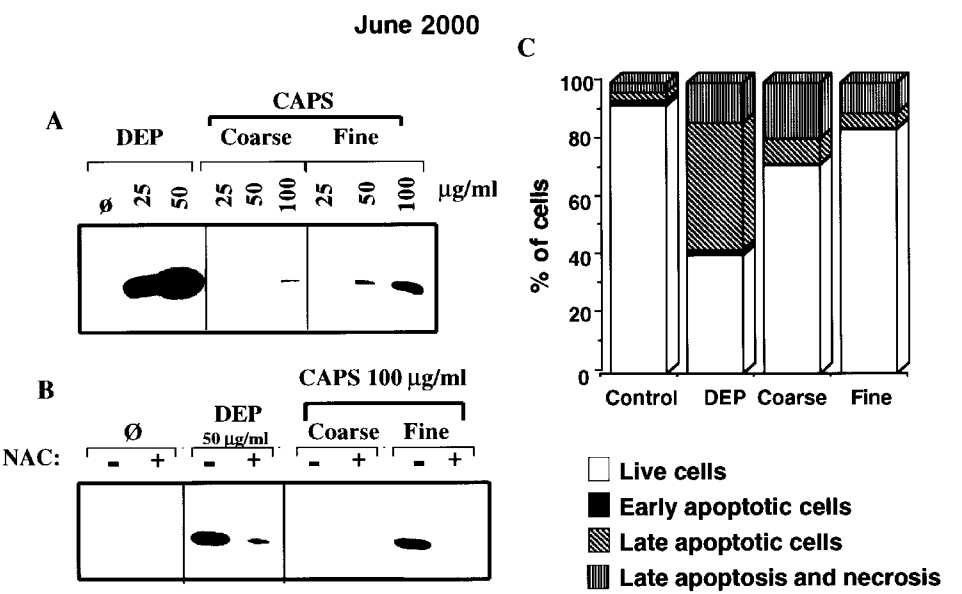

FIGURE 10. Comparison of coarse and fine PM effects on HO-1 expression and cytotoxicity in June 2000. (A) Effects of DEP as well as coarse and fine PM on HO-1 expression. RAW 264.7 were exposed to the indicated final concentrations of these particles for 16 h. (B) Effect of NAC, 20 m*M*, on HO-1 expression by DEP and CAPS used at the indicated concentrations. (C) Effects of DEP, coarse and fine PM, in the annexin V/PI staining protocol as described in Figure 5. Cells were incubated with 400 µg/ml of each of these particles for 18 h.

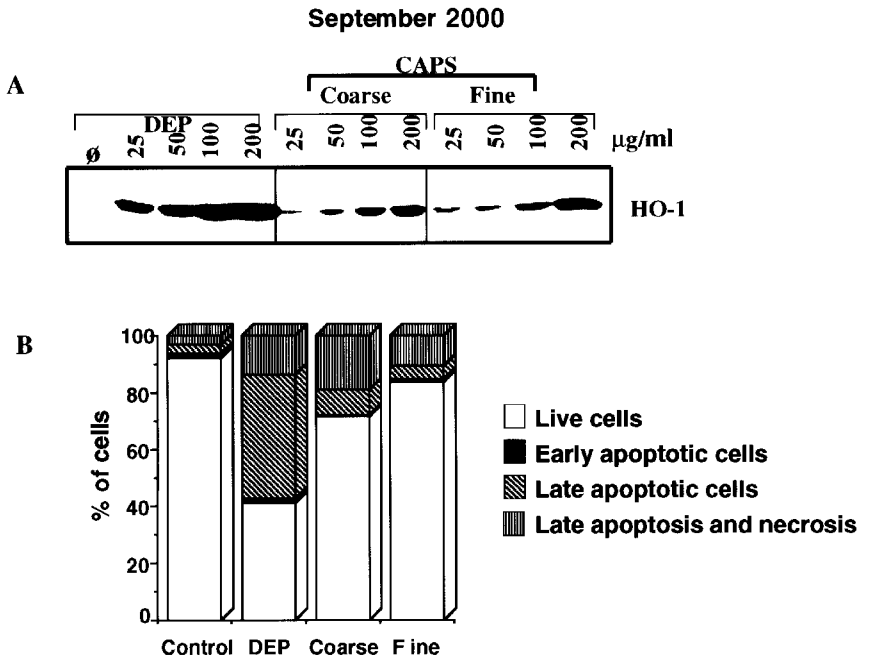

FIGURE 11. Comparison of coarse and fine PM effects on HO-1 expression and cytotoxicity in September 2000. (A) Western blot showing HO-1 expression after incubation with DEP and CAPS for 16 h. (B) Annexin V/PI staining of RAW 264.7 cell pellets incubated together with 300 μg/ml DEP, coarse or fine PM for 18 h.

CO in the expired air (Horvath et al., 1998; Choi & Alam, 1996). HO-1 is also expressed in the airways of asthmatics, and acts as a marker for airway inflammation in this disease (Choi & Alam, 1996).

The molecular basis for the high sensitivity of the HO-1 promoter to oxidative stress depends on the transcriptional activation of a number of antioxidant response elements (ARE), also known as the stress-induced response elements (StRE), in the promoter of that gene (Prestera et al., 1995). These ARE/StREs express homology to the consensus binding sites for the Nrf, AP-1, and small Maf transcription factors, and are regulated by several members belonging to those families (Alam et al., 1999, 2000; Kataoka et al., 1994). It has now been established that Nrf-2 positively regulates HO-1 expression in the mouse lung as well as tissue culture cells (Alam et al., 1999; Chan & Kan, 1999). One explanation for the effects of oxidative stress on this transcription factor is the release of Nrf-2 from the cytosol, where it is sequestrated by a binding protein, to the nucleus (Itoh et al., 1999).

Although preliminary, the correlation between the generation of oxidative stress and the relative abundance of the OC fraction suggests that PAHs and their oxy derivatives may be involved in the biological effects of CAPS.

In this regard, it is known that metabolic conversion of PAHs yields quinones, some of which can redox cycle to yield ROS (Penning et al., 1999; O'Brien, 1991; Monks et al., 1992). In addition, DEP contain oxy-PAH derivatives that may directly participate in ROS generation (Li et al., 2000; Shuetzle et al., 1981). This notion is confirmed by previous data from our laboratory, which show that aromatic and polar compounds fractionated from DEP by silica gel chromatography can induce HO-1 expression (Li et al., 2000). Some of those results were reproduced in Figure 9. Chemical analysis demonstrated that the aromatic and polar fractions are enriched for PAHs and quinones, respectively (Li et al., 2000). While the same fractionation still needs to be carried out for coarse and fine particles, our current data set demonstrates good correlation between PAH content of these CAPS and their ability to induce HO-1 expression in macrophages (Figures 9–12). While fine particulates consistently induced HO-1 expression in association with a high PAH content, coarse particles only induced in HO-1 expression when the PAH rose during the fall and winter months (Figures 12 and 13). We need to point out, however, that the coarse particle mode collected in September 2000 induced HO-1 expression without an increase in the OC ratio above the mean for the study period (Figures 7 and 11). That

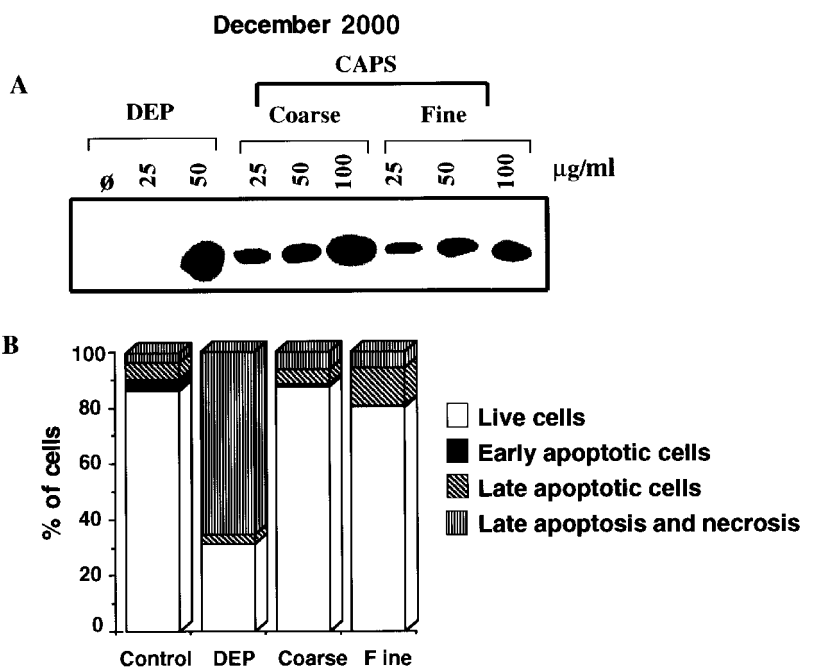

FIGURE 12. Comparison of coarse and fine PM effects on HO-1 expression and cytotoxicity in December 2000. (A) Western blot showing HO-1 expression after incubation with DEP and CAPS for 16 h. (B) Annexin V/PI staining of RAW 264.7 cell pellets incubated together with 250 µg/ml DEP, coarse or fine PM for 18 h.

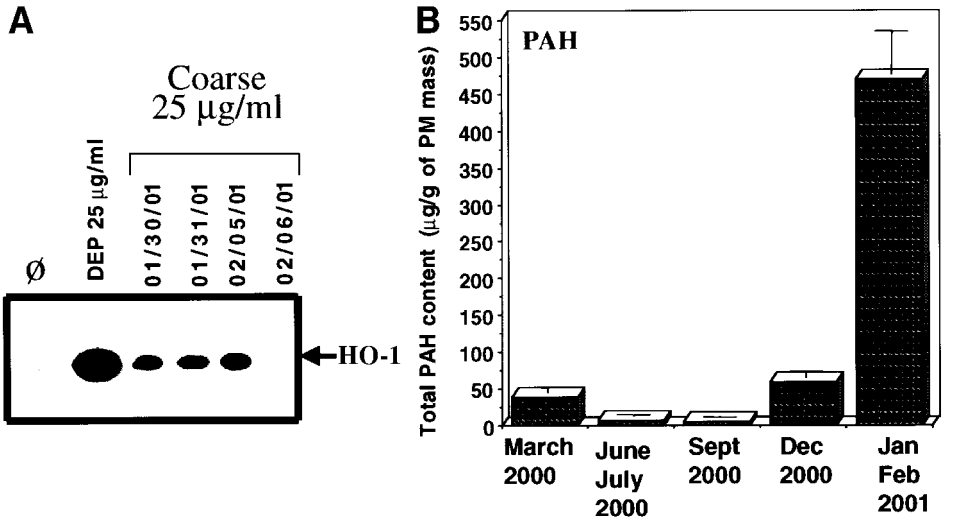

FIGURE 13. Increased PAH content of coarse PM during the winter months correlates with the ability of these particles to induce HO-1 expression. (A) Additional coarse PM samples were collected on the dates shown and used for conducting HO-1 expression as described in Figures 9–12. (B) We compared average PAH concentrations for the months shown. This represents three samples in March 2000, three in June/July 2000, two in September 2000, two in December 2000, and four in January/February 2001. Error bars represent standard deviations. The PAH levels were statistically significantly higher during the winter than the summer months: Comparison of December 2000 with June/July 2000 yielded $p < .001$, while comparison of January/February 2001 with June/July 2000 yielded $p < .001$.

suggests that other particle components, such as transition metals, may contribute to the generation of oxidative stress (Ghio & Samet, 1998; Gilmour et al., 1996). Although coarse particles generally express a higher metal content than fine particles (Figure 8A), we could not interfere with HO-1 expression by addition of metal chelators to the culture medium (not shown). However, we could interfere with HO-1 expression by the thiol antioxidant *N*-acetylcysteine (Figure 10). This is interesting from the perspective that thiol antioxidants, in addition to acting as radical scavengers and precursors for glutathione synthesis, may covalently complex to quinones via 1,4-Michael addition reactions.

The elevated PAH content of coarse PM during the fall and winter seasons may be directly related to the drop in ambient temperatures during those months. For instance, the average sampling temperatures during January/February 2001 were in the 12–15°C range, compared to 20–25°C during March 2000 and >30°C during June/July 2000. According to the adsorptive partitioning model of Junge (1977) and Pankow (1987), it is possible to predict the gas–particle distribution of semivolatile organics according to the formula $\psi = c\theta/(P_L^\circ + c\theta)$, where ψ represents the particle bound fraction, θ is the particle surface area per volume of air, P_L° is the liquid

vapor pressure of PAHs, and c is a constant dependent on heat condensation and surface properties. The vapor pressure increases exponentially with increasing ambient temperatures, and as a result the gas-to-particle partitioning of any specific PAHs would favor the gas phase. This equation also indicates that the particulate association of specific PAHs will increase as P_L° decreases due to a drop in ambient temperature. It is also important in the partitioning model to consider the molecular size of the PAH compounds. PAHs with molecular weight (MW) from 128 (NAP) up to 202 (FLT and PYR) are generally <10% particle associated, while CRY (MW 228) is <40% and BBA (MW 228) >60% particle associated. PAHs with MW > 252 are predominantly particle associated (Junge & Pankow, 1977).

There are explanations other than the differences in OC and PAH content to explain the biological differences between of coarse and fine particles. One possibility is differences in their rates of phagocytosis. Although there is no readily available methodology to address this question at present, it is important to consider that particle number and surface area may be alternative or better criteria than particle mass on which to base toxicological results. It is important to stress, therefore, that our current data showing a correlation between OC mass and HO-1 inducibility must be seen as preliminary, and needs to independently confirmed.

The establishment of a stratified oxidative stress model could prove a valuable tool for the evaluation of the biological and clinical effects of PM, including DEP and CAPS. Although the biological effects of PM can be mediated independent of oxidative stress, such as described for particles alone (Oberdörster, 2001), the oxidative stress hypothesis allows one to dissect the biological effects of PM in a systematic fashion. Moreover, the stratified nature of the response dictates a selection of endpoints relevant to the levels of exposure. For instance, under the experimental conditions described by Nightingale et al. (2000), increased CO levels in the expired air was the most sensitive endpoint for discerning DEP effects in the lung. This agrees with our findings that HO-1 is a sensitive in vitro marker for oxidative stress (Figures 3, 9–12). While the effects on BAL neutrophil counts were less dramatic than the effects on CO levels (Nightingale et al., 2000), DEP delivery to the nose of atopic individuals was able to elicit a vigorous inflammatory response (Diaz-Sanchez et al., 1997). This pronounced inflammatory effect in the nose likely reflect higher exposure levels, as well as the possibility that preexisting allergic inflammation may contribute to oxidative stress effects. These findings suggest that the endpoint measurements should be carefully titrated to the level of exposure and the level of oxidative stress that may be induced or already exists in the exposed tissue. In the planning of in vivo experiments, it is also important to consider that if the levels of oxidative stress become excessive, cytotoxic effects (Figure 5) may override and conceal the proinflammatory effects of the PM. One possibility is that apoptosis of macrophages and participating immune cells may destroy the cellular elements that are required for IgE production and generation of eosino-

philic inflammation. Proapoptotic effects may also be relevant to airway hyperreactivity independent of the effects of allergic inflammation (Nel et al., 2001). Apoptosis and desquamation of epithelial cells may expose irritant receptors that regulate bronchial smooth muscle activity.

Another value of the oxidative stress theory is that it may assist in the identification of human subsets that are more susceptible to the adverse health effects of PM. An example is HO-1 expression. This enzyme has potent antioxidant and cytoprotective effects in the lung (Choi et al., 1996). It is noteworthy that a polymorphism has been described in the HO-1 promoter that determines gene expression in the presence of ROS (Yamada et al., 2000). Moreover, it has been demonstrated that male smokers with a poorly responsive HO-1 promoter have a higher rate of emphysema than male smokers with a more inducible HO-1 gene (Yamada et al., 2000). The same paradigm may also apply to other antioxidant defense or chemical detoxification pathways that play a role in defending against the adverse biological effects of PM. The induction of these defense pathways may also explain the phenomenon of tolerization, which leads to a blunting of adverse PM effects upon reexposure.

Traditionally, particle collection for in vitro tests has been conducted by using collection substrates such as filters or impactors. Particles collected on filters are subsequently extracted from the substrates and administered into in vitro culture media, either directly or after lyophylization of the solvent. This process suffers from several shortcomings, including inefficient particle extraction from the substrate, and variable losses of potentially toxic semivolatile PM constituents and of biologically active components of airborne PM (Willeke et al., 1998). In addition, a recent study by Dick et al. (2000) showed that components of filters used to collect particles could contaminate the preparation and interfere with biological investigations. Particle collection using liquid impingers has been shown to be advantageous over the traditional filtration or impaction methods for collection of airborne particles, because impingers are not easily overloaded (Willeke et al., 1998) and because impingement eliminates the need for elaborate extraction procedures (Zucker et al., 2000).

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